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SCIENCE DIRECT
Developmental Biology 290 (2006) 470 – 481
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Genomes & Developmental Control

FGF8, *Wnt8* and *Myf5* are target genes of Tbx6 during anteroposterior specification in *Xenopus* embryo

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Received for publication 21 March 2005, revised 17 October 2005, accepted 14 November 2005
Available online 15 December 2005

Abstract

The T-box transcription factor Tbx6 is required for somite formation and loss-of-function or reduced activity of Tbx6 result in absence of posterior paraxial mesoderm or disorganized somites, but how it is involved in a regulatory hierarchy during *Xenopus* early development is not clear. We show here that *Tbx6* is expressed in the lateral and ventral mesoderm of early gastrula, and it is necessary and sufficient to directly and indirectly regulate the expression of a subset of early mesodermal and endodermal genes. Ectopic expression of Tbx6 inhibits early neuroectodermal gene expression by strongly inducing the expression of posterior mesodermal genes, and expands the mesoderm territory at the expense of neuroectoderm. Conversely, overexpression of a dominant negative Tbx6 mutant in the ventral mesoderm inhibits the expression of several mesodermal genes and results in neural induction in a dose-dependent manner. Using a hormone-inducible form of Tbx6, we have identified *FGF8*, *Xwnt8* and *XMyf5* as immediate early responsive genes of Tbx6, and the induction of these genes by Tbx6 is independent of Xbra and VegT. These target genes act downstream and mediate the function of Tbx6 in anteroposterior specification. Our results therefore identify a regulatory cascade governed by Tbx6 in the specification of posterior mesoderm during *Xenopus* early development.

Keywords: T-box; Tbx6; FGF8; Wnt8; Myf5; Myogenesis; Paraxial mesoderm; Anteroposterior patterning; Neural induction; *Xenopus*

Introduction

Members of the *T-box* gene family play central roles for the development of mesoderm. *T-box* genes are a family of developmental regulators with more than 20 members recently identified in invertebrates and vertebrates (reviewed by Papaioannou, 1997; Smith, 1999). Mutations in *T-box* genes have been found to cause several human diseases (Bamshad et al., 1997; Li et al., 1997; Basson et al., 1997). The founding family member, *Brachyury* (or *T*), was originally identified by mutation in the mouse (Dobrovolskaïa-Zavadskaïa, 1927) and then cloned (Herrmann et al., 1990). All of the *T-box* genes whose functions have been studied are essential for early development. *Brachyury* is required for mesoderm specification and morphogenetic movements of gastrulation (Conlon et al., 1996; Smith et al., 1991, 2000). *Eomesodermin* (*eomes*) is implicated in mesoderm development both in *Xenopus* and in mouse (Ryan et al., 1996; Russ et al., 2000). *Xenopus VegT* is a maternal mRNA and is zygotically expressed in the presumptive mesoderm and is restricted to the lateral and ventral mesoderm by the end of gastrulation (Zhang and King, 1996; Lustig et al., 1996; Stennard et al., 1996; Horb and Thomsen, 1997). Depletion experiments show that *VegT* function is required for endoderm and mesoderm development by regulating TGFβ family signaling molecules (Zhang et al., 1998; Clements et al., 1999; Kofron et al., 1999; Xanthos et al., 2001). In chick limb, Tbx5 and Tbx4 have been shown to regulate the expression of *Wnt* and *FGF* genes (Ng et al., 2002; Takeuchi et al., 2003), raising the possibility that *T-box* genes play a crucial role in gene regulatory hierarchy during vertebrate myogenesis.

Among different *T-box* genes, *Tbx6* is expressed in primitive streak, the paraxial mesoderm, and the tail-bud in different species (Chapman et al., 1996; Hug et al., 1997; Griffin et al., 1998; Uchiyama et al., 2001). It plays an important role in the

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0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.11.020

formation of posterior paraxial mesoderm. In *Tbx6* null mutant mouse embryos, posterior paraxial mesoderm develops as neural tissues, suggesting that it is essential for the formation of posterior somitic mesoderm (Chapman and Papaioannou, 1998). In addition, it has been shown recently that the mouse rib-vertebrae mutation is a hypomorphic *Tbx6* allele (Watabe-Rudolph et al., 2002; White et al., 2003). This mutation affects somite formation, morphology and patterning, and leads to malformations of the axial skeleton such as split vertebrae and neural arches, and fusions of adjacent segments (Theiler and Varnum, 1985; Beckers et al., 2000; Nacke et al., 2000). Analyses of these mutations also reveal that Tbx6 interacts genetically with Notch pathway genes (White et al., 2003; Hofmann et al., 2004). Together, both loss-of-function and reduced activity of Tbx6 suggested that it occupies a key position in posterior paraxial mesoderm formation (Chapman et al., 1996; Chapman and Papaioannou, 1998; White et al., 2003).

Despite this important function, little is known about its regulation and its interaction with other factors involved in anteroposterior patterning and myogenesis. Since *Tbx6* is expressed in the posterior region in all vertebrates, another unanswered question is whether and how it is involved in the patterning of anteroposterior axis. In this report, we analyzed the activity of Tbx6 in mesoderm and neural induction, as well as in the patterning of anteroposterior axis. Our results show that Tbx6 regulates, both directly and indirectly, the expression of a subset of early mesodermal and endodermal genes. It protects posterior mesoderm from neural induction and directly interacts with *FGF8*, *Xwnt8* and *XMyf5* in anteroposterior patterning and in regulating *XMyoD* expression.

Materials and methods

Plasmid constructs and morpholino oligonucleotides

The *pCS2-Tbx6VP16* and *pCS2-Tbx6EnR* constructs were kindly provided by Dr. H. Uchiyama. A hormone-inducible construct of *Tbx6VP16* cloned in *pSP64T* vector (*pSP64T-Tbx6VP16-GR*) was obtained through fusion of *Tbx6VP16* with the ligand-binding domain of the human glucocorticoid receptor (amino acids 512–777, with an initiation methionine added to the amino-terminus). *pSP64T-Xbra*, *pSP64T-XbraEnR* and *pSP64T-VegT* were from Dr. J. Smith. *pSP35T-chordin* was from Dr E. De Robertis. *pSP6nucβgal* was from Dr. R. Harland and *pSP64T-XFD* was from Dr. M. Kirschner. *pCS2-FGF8* and *pCS2-Tbx6* were obtained by PCR amplification and cloned into the *pCS2* vector. *pCS2-VegTEnR* was also obtained by PCR amplification of *VegT* DNA binding domain (amino acids 1 to 284) and cloned inframe with the *Drosophilia Engrailed* repressor domain (amino acids 2 to 298). Synthetic capped mRNA was made by in vitro transcription as described (Djiane et al., 2000). The morpholino oligonucleotides for *XMyf5* (5′-ACCATCTCCATTCTGAATAGTGCTG-3′), *Xwnt8* (5′-AAAGTGGTGTTTTGCATGATGAAGG-3′) and control sequence were from Gene Tools, and suspended in sterile water at a concentration of 2 mg/ml.

Xenopus embryos and tissue explants

Xenopus eggs were obtained from females injected with 500 IU of human chorionic gonadotropin (Sigma), and artificially fertilized with minced testis. Eggs were dejellied with 2% cysteine hydrochloride (pH 7.8) and kept in 0.1× modified Barth solution (MBS) to appropriate stages (Nieuwkoop and Faber, 1967) for further manipulations. Microinjections of embryos at different stages were done in 0.1× MBS containing 3% Ficoll-400. After injections, embryos were kept in 3% Ficoll-400 solution for 3 h and then cultured in 0.1× MBS until they reached appropriate stages. After injection of *Tbx6VP16-GR* mRNA (20 to 100 pg), whole embryos or animal cap explants were cultured to different stages and then incubated in 10 μM of dexamethasone in the presence or absence of 10 μM of the protein synthesis inhibitor, cycloheximide.

In situ hybridization and cell lineage tracing

Whole-mount in situ hybridization was performed according to standard protocol (Harland, 1991). *Tbx6* probe was a 1.5 kb cDNA insert cloned in *pBluescript* vector and was obtained by systematic sequencing of a gastrula cDNA library. It was linearized with *Not*I and transcribed with T7 RNA polymerase to generate antisense RNA. Probes for *Xbra*, *Xwnt8*, *XMyf5*, *XMyoD* and *myosin light chain* were described previously (Shi et al., 2002). *Sox3* probe was provided by Dr. H. Grunz and *Sox17α* was from Dr. H. Woodland. They were linearized with *Eco*RI or *Sma*I, respectively, and transcribed with T7 RNA polymerase. *FGF8* probe was linearized from the *pCS2* expression plasmid with *Bam*HI and transcribed with T3 RNA polymerase. *LacZ* mRNA was injected as a cell lineage tracer and β-galactosidase staining was performed as described (Vize et al., 1991).

RT-PCR

Extraction of total RNA from whole embryo or animal cap explants was performed using guanidine isothiocyanate/phenol followed by LiCl precipitation to remove genomic DNA and polysaccharides. RNA samples were further treated with RNAse-free DNAse I (Roche) and were reverse-transcribed using 200 units SuperScript reverse transcriptase (Life Technologies). PCR primers for *Xbra*, *XMyoD*, *XMyf5*, *Xwnt8*, *chordin* and *ornithine decarboxylase* (*ODC*) were as described (Shi et al., 2002). Other primers are as follows: *Otx2* (F: 5′-GGATGGATTTGTTGCACCAGTC-3′, R: 5′-CACTCTCCGAGCTCACTTCTC-3′), *XAG1* (F: 5′-CTGACTGTCCGATCAGAC-3′, R: 5′-GAGTTGCTTCTCTGGCAT-3′), *N-CAM* (F: 5′-CACAGTTCCACCAAATGC-3′, R: 5′-GGAATCAAGCGGTACAGA-3′), *muscle actin* (F: 5′-GCTGACAGAATGCAGAAG-3′, R: 5′-TTGCTTGGAGGAGTGTGT-3′), *Hoxb9* (F: 5′-TACTTACGGGCTTGGCTGGA-3′, R: 5′-AGCGTGTAACCAGTTGGCTG-3′), *Xcad2* (F: 5′-ATAACAATCCGCAGGAAG-3′, R: 5′-TTGATGATGGAGATACCAAG-3′), *Mespo* (F: 5′-CTTACTACTGATGGAGACTC-3′, R: 5′-AATCGATAGCCAACCTCA-3′), *FGF8* (F: 5′-ATCCAACTGGCAACTGAGC-3′, R: 5′-AGAAATTACTGTCATAGTCC-3′), *FGF3* (F: 5′-TCGGCCATGCCACAATG-3′, R: 5′-AGTTTGCACCCCTCACTGTCC-3′), *Sizzled* (F: 5′-CAAACCTGATGGGACACACTAAC-3′, R: 5′-GCTTAGGCAATTCTTTTATAGGAG-3′), *goosecoid* (F: 5′-ACAACTGGAAGCACTGGA-3′, R: 5′-TCTTATTCCAGAGGAACC-3′), *En2* (F: 5′-CGGAATTCATCAGGTCCGACAATC-3′, R: 5′-GCGGATCCTTTGAAGTGGTCGCG-3′), *Sox17α* (F: 5′-GGACGAGTGCCAGATGATG-3′, R: 5′-CTGGCAAGTACATCTGTCC-3′), *Delta 1* (F: 5′-AATGAATAACCTGGCCAACTG-3′, 5′-GTGTCTTTTGACGTTGAGTAG-3′. One-twentieth of the reverse-transcribed cDNA was used for PCR amplification in a reaction mixture containing 1 μCi of [α-^{32}P]dCTP (ICN Pharmaceuticals). PCR products were resolved on a 5% non-denaturing polyacrylamide gel and visualized and quantified by a Phospho-Imager (BioRad).

Histology

Embryos or tissue explants were fixed in 3.7% formaldehyde buffered in MOPS. Histological sections were cut after embedding the embryos or explants in polyethylene glycol distearate.

Results

Tbx6 in dorsoventral and anteroposterior patterning

In *Xenopus* embryo, *Tbx6* is shown to be expressed in the posterior paraxial mesoderm as in other vertebrates (Uchiyama